

CONCISE COMMUNICATION

Effects of Clinically Used Antioxidants in Experimental Pneumococcal Meningitis

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Reactive oxygen intermediates mediate brain injury in bacterial meningitis. Several antioxidant drugs are clinically available, including N-acetylcysteine (NAC), deferoxamine (DFO), and trylizad-mesylate (TLM). The present study evaluated whether these antioxidants are beneficial in a model of pneumococcal meningitis. Eleven-day-old rats were infected intracranially with *Streptococcus pneumoniae* and randomized to intraperitoneal treatment every 8 h with NAC (200 mg/kg), DFO (100 mg/kg), TLM (10 mg/kg), or saline (250 μ L). TLM-treated animals showed a significantly reduced mortality compared with controls ($P < .03$). Meningitis led to extensive cortical injury at 22 ± 2.2 h after infection (median, 14.6% of cortex; range, 0–61.1%). Injury was significantly ($P < .01$) reduced to 1.1% (range, 0–34.6%) by NAC, to 2.3% (range, 0–19.6%) by DFO, and to 0.2% (range, 0–36.9%) by TLM (the difference was not significant among the 3 groups). None of the drugs reduced hippocampal injury. Thus, several clinically used antioxidants reduced cortical injury in experimental pneumococcal meningitis.

Reactive oxygen intermediates (ROIs), a family of molecules derived from the partial reduction of molecular oxygen, have been implicated as mediators of neuronal damage in bacterial meningitis [1–3]. ROIs are released by the host from stimulated granulocytes, macrophages, and microglia, and *Streptococcus pneumoniae* has been shown to utilize pyruvate oxidase to produce ROIs. Because of their free radical nature (i.e., the presence of an unpaired electron) and loss of spin restriction, ROIs react avidly with organic molecules and are cytotoxic by oxidizing membrane phospholipids, proteins, nucleic acids, and nucleotides.

A number of factors make the central nervous system particularly susceptible to oxidative damage. The brain receives ~20% of the cardiac blood output and thus has a high oxygen tension, and brain cell membranes are rich in polyunsaturated fatty acids that can be oxidized by ROIs. Brain tissue has rel-

atively low levels of endogenous antioxidant defense systems. In previous studies based upon a model of bacterial meningitis caused by group B streptococci, we localized the generation of ROIs to cells of the subarachnoid and ventricular inflammation and to the cerebral vasculature, the site of blood-brain barrier breakdown [1].

In an infant rat model of meningitis due to group B streptococci, the spin-trapping agent α -phenyl-tert-butyl nitron significantly attenuated neuronal damage [1]. Other antioxidants attenuated a number of early events associated with bacterial meningitis, such as early increases in cerebral blood flow, intracranial pressure, brain water content, cerebrospinal fluid (CSF) white blood cell count, and pial arteriolar vasodilatation [4–6].

For the present study, we used an established rat model of experimental neonatal meningitis due to *S. pneumoniae*, which leads to neuronal damage [7, 8]. The model shows features identical to those described in neonates and infants with neonatal meningitis. Two distinct forms of neuronal injury have been documented: extensive cortical injury and apoptotic injury to the dentate gyrus of the hippocampus [1].

In this study, we examined the effect of 3 agents that interfere with ROI production at different stages (figure 1). N-acetylcysteine (NAC), which increases endogenous antioxidant defenses (glutathione), is clinically used for paracetamol intoxication [4, 9]. Deferoxamine (DFO), an iron-chelating agent, decreases the concentration of oxidative radicals by inhibiting the catalyzation of radical production by iron [6, 10]. Trylizad-mesylate (TLM), which protects cell membranes from peroxidation, is therapeutically used to prevent brain injury in patients

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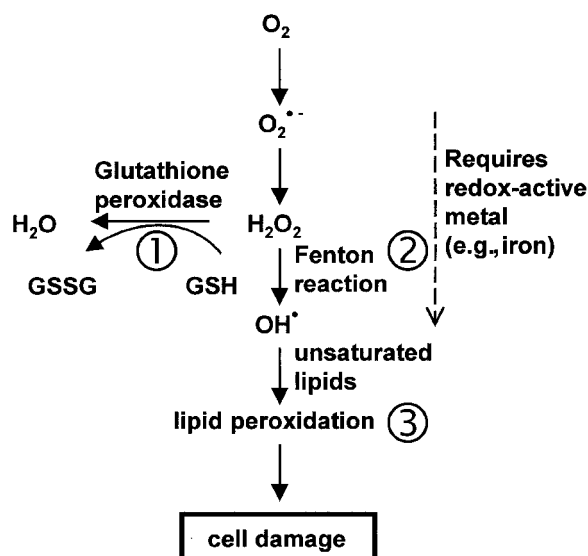


Figure 1. Formation of reactive oxygen species and antioxidant mechanisms in biologic systems. Superoxide ($O_2^{\cdot -}$), formed by a one-electron reduction of molecular O_2 , is converted to H_2O_2 by dismutation and further to OH^{\cdot} by the Cu^+/Fe^{2+} -catalyzed Fenton reaction. The resultant free radical damage to lipid (peroxidation), proteins, and DNA leads to various forms of cell injury. The major antioxidant enzymes are superoxide dismutase, catalase, and glutathione peroxidase. GSH, reduced glutathione; GSSG, oxidized glutathione. As numbered in the figure, the antioxidants used for therapy were (1) N-acetylcysteine (NAC), which increases the endogenous antioxidant glutathione; (2) deferoxamine (DFO), which decreases the concentration of oxidative radicals by neutralizing iron; and (3) trylazad-mesylate (TLM), which inhibits lipid peroxidation.

with subarachnoid-space bleeding [11]. In the present study, we studied the 3 drugs to explore whether these clinically available antioxidants should be tested in clinical trials as adjunctive therapy for bacterial meningitis.

Material and Methods

Model of meningitis. Nursing Sprague-Dawley rats were infected on postnatal day 11 by direct intracisternal injection, with a 32-gauge needle, of 10 μ L of a saline solution containing *S. pneumoniae* (serogroup 3), as described elsewhere [7, 8]. The inoculum size was $\log_{10} 6.5 \pm 0.35$ cfu/mL. Eighteen hours after infection, the rats were weighed and assessed clinically for their ambulatory activity and their ability to right themselves. The occurrence of spontaneous seizures was documented during the following 2 h. CSF (1–30 μ L) was obtained by puncture of the cisterna magna. To document meningitis, 10 μ L of CSF was cultured quantitatively. Starting 18 h after infection, the animals were treated subcutaneously with 100 mg/kg ceftriaxone. Those surviving 24 h of infection were killed with an overdose of pentobarbital (100 mg/kg intraperitoneally). Animals who died unobserved were excluded from the histopathologic evaluation.

Therapeutics. Three molecular classes of antioxidant agents were used: NAC (Fluimucil, 20% infusion solution; Inpharzam,

Cadempino, Switzerland), DFO (Sigma, Division of Fluka Chemie, Buchs, Switzerland), and TLM (Freedox, 150-mg sterile solution; Pharmacia and Upjohn, Dübendorf, Switzerland). Therapy was begun at the time of infection and was given every 8 h. The NAC group ($n = 24$) received 200 mg/kg, the DFO group ($n = 25$) received 100 mg/kg, and the TLM group ($n = 25$) received 10 mg/kg. As a control group, 30 rats received saline. The doses used in this study correspond to doses from published studies using rat models of experimental meningitis for NAC [4], a rat model of pneumonia for DFO [10], and a rat model of the “shaken-baby syndrome” for TLM [11].

Histopathology. For histopathologic examination, killed animals were perfused with 4% paraformaldehyde in PBS, and 12 coronal brain sections per animal were evaluated for neuronal injury to the cortex and hippocampus, as described elsewhere [7, 8]. The area of cortical brain damage was expressed as the mean value per animal of the percentage of the total cortex in each of the 12 sections. Apoptotic death of neurons in the dentate granule cell layer of the hippocampus was scored, and an averaged score per animal was calculated from all evaluated sections. All histopathologic evaluations were done by an investigator blinded to the clinical, microbiologic, and treatment data of the respective animal.

Measurement of tumor necrosis factor- α (TNF- α). The concentration of TNF- α in the CSF samples was measured by ELISA (Cytoscreen Rat Tumor Necrosis Factor-Alpha Ultra Sensitive; BioSource International, Camarillo, CA) [7]. CSF samples (10 μ L) from infected animals of each study group (NAC, $n = 13$; DFO, $n = 18$; and TLM, $n = 15$) and from controls ($n = 15$) were sampled 18 h after infection and immediately centrifuged, and the supernatant was frozen at -80°C until analyzed, following the manufacturer’s instructions.

Statistics. Continuous data were presented as mean \pm SD, categorical data as median and range. Data not normally distributed were analyzed by the Kruskal-Wallis test, followed by the Mann-Whitney test for pairwise comparison. Proportions between groups were analyzed using Fisher’s exact test. Survival curves were analyzed by Kaplan-Meier analysis for survival data.

Results

Effect of antioxidants on clinical parameters of meningitis. All infected animals had meningitis, as evidenced by lethargy or obtundation and by positive bacterial CSF titers at 18 h after infection. Treatment with NAC and TLM showed no effect on CSF bacterial titers (table 1). Animals in the DFO treatment group had lower CSF bacterial titers than animals in all other groups, which was likely a consequence of growth inhibition by iron limitation in the CSF. Treatment with antioxidants did not significantly affect the occurrence of seizures (table 1). However, compared with controls, TLM-treated animals showed a significant ($P < .03$) attenuation of spontaneous mortality, while treatment with NAC and DFO had no significant beneficial effect (table 1).

Effect of antioxidants on CSF concentration of TNF- α . The CSF concentrations of TNF- α were measured as an indicator of inflammation. There was no significant difference ($P > .05$)

Table 1. Effect of antioxidant treatment on clinical parameters of meningitis.

| Group | CSF bacterial titer, log ₁₀ cfu/mL | Occurrences of seizures, % | Spontaneous death, % | TNF- α in CSF, ng/mL | Cortical injury, % | Hippocampal injury score |
|----------|--|-------------------------------|-------------------------|--------------------------------|--------------------------------|-----------------------------|
| Controls | 8.20 \pm 0.54 (28) | 50.0 (28) | 71.4 (28) | 4.8 \pm 2.9 (15) | 14.6 [0–61.1] (28) | 0.3 [0–1.08] (18) |
| NAC | 8.17 \pm 0.57 (23) | 30.4 (23) | 48.7 (23) | 4.9 \pm 2.6 (13) | 1.1 [0–34.6] ^a (23) | 0.3 [0–1.04] (23) |
| DFO | 7.86 \pm 0.48 ^b (22) | 45.5 (22) | 59.1 (22) | 5.8 \pm 2.1 (18) | 2.3 [0–19.6] ^c (22) | 0.5 [0–1.33] (20) |
| TLM | 8.12 \pm 0.58 (25) | 44.0 (25) | 32.0 ^d (25) | 4.9 \pm 2.0 (15) | 0.2 [0–36.9] ^e (25) | 0.3 [0–1.17] (22) |

NOTE. CSF, cerebrospinal fluid; TNF- α , tumor necrosis factor- α ; NAC, N-acetylcysteine; DFO, deferoxamine; TLM, trylizad-mesylate. CSF bacterial titer and TNF- α in CSF are presented as mean \pm SD; cortical injury and hippocampal injury scores are presented as median [range]. Numbers in parentheses = *n*.

^a *P* < .02 vs. controls.

^b *P* < .03 vs. controls.

^c *P* < .01 vs. controls.

^d *P* < .03 vs. controls.

^e *P* < .01 vs. controls.

between any of the experimental groups at 18 h after infection (table 1).

Effect of antioxidants on neuronal injury. Two distinct forms of neuronal injury were identified in this experimental model of bacterial meningitis. The neurons of the cortex showed morphologic signs that were compatible with necrosis, such as marked swelling of the cell body and loss of cellular demarcation and cytoarchitecture [1]. The injury patterns in the hippocampus were compatible with apoptosis. The occurrence of apoptosis in the dentate gyrus in this model was confirmed in a previous study by positive-terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) of fragmented DNA in the dentate gyrus but not in the cortex [1].

All 3 antioxidants decreased the extent of cortical injury. The decrease varied from a median of 14.6% (range, 0–61.1%) in 28 control rats to 1.1% (range, 0–34.6%) in 23 NAC-treated rats (*P* < .02), to 2.3% (range, 0–19.6%) in 22 DFO-treated animals (*P* < .01), and to 0.2% (range, 0–36.9%) in 25 rats treated with TLM (*P* < .01). There were no significant differences in neuroprotective efficacy among the 3 antioxidant therapies (table 1).

In contrast, antioxidant treatment had no effect on the extent of apoptosis in the hippocampus. The median injury score was 0.3 (range, 0–1.04) for the NAC group (*n* = 23), 0.5 (range, 0–1.33) for the DFO group (*n* = 20), 0.3 (range, 0–1.17) for the TLM group (*n* = 22), and 0.3 (range, 0–1.08) for the control group (*n* = 18; table 1).

Discussion

In the present study, we used an infant rat model to evaluate the effect of clinically used antioxidants on the clinical and neuropathological outcomes during pneumococcal meningitis. We demonstrated that 3 classes of antioxidants (NAC, DFO, and TLM) that interfere with the harmful effects of ROIs at different levels exert a neuroprotective effect in the cortex (figure 1). Despite this beneficial effect, we found no reduction of the inflammatory response (i.e., CSF concentration of TNF- α) or

of hippocampal apoptosis as a result of treatment with any of the 3 compounds.

We tested the effect of NAC because it has been used as an antioxidant in a variety of experimental and clinical conditions [4, 9]. At present, NAC is known to have 2 modes of action: direct scavenging of free radicals and augmentation of intracellular levels of glutathione [4, 9]. NAC has proven efficacy as an antioxidant in humans and animals with various diseases, including experimental bacterial meningitis, in which it significantly attenuated the increase in brain water content, intracranial pressure, and CSF pleocytosis 24 h after infection [4]. The results of the present study expand these findings by showing a beneficial effect of the drug on cortical injury in neonatal experimental meningitis. The basis for this beneficial effect is not completely clear. We have previously documented that ROIs are generated at the level of the cerebral vasculature and that cerebral blood flow improved upon treatment with the radical scavenger α -phenyl-tert-butyl nitrone [1]. Thus, in keeping with the evidence that ischemia contributes to cortical injury in this model, the neuroprotection of NAC in the cortex might be mediated by preventing disturbances of cerebral blood flow [3, 4, 8].

An important factor that contributes to the central nervous system's susceptibility to oxidative damage is its rich content of iron. Extensive experimental support exists for the early occurrence and pathophysiologic importance of oxygen radical formation and cell membrane lipid peroxidation in the injured nervous system. The radical-initiated peroxidation of neuronal, glial, and vascular cell membranes and myelin is catalyzed by free iron released from hemoglobin, transferrin, and ferritin by lowered tissue pH and oxygen radicals. Thus, a disruption of the normal iron homeostasis during bacterial meningitis may lead to an increase in oxygen free radical generation and consecutive tissue damage.

DFO was used to evaluate the role of iron in our model of meningitis. Compounds such as DFO are excellent chelating agents of free Fe³⁺ and are currently being used clinically to treat iron-overload patients. In our study, DFO attenuated cortical injury. One possible mechanism for this beneficial effect

is through inhibition of the $\text{Cu}^+/\text{Fe}^{2+}$ -catalyzed Fenton reaction. This would reduce the generation of hydroxyl radicals as well as that of other reactive products, thus leading to reduced oxidative damage in the brain [2]. However, more studies are needed to delineate the exact mechanisms by which DFO exerts its protective role against ischemia-induced cortical injury in this model.

The 21-aminosteroid TLM has been shown to be a potent inhibitor of lipid peroxidation and to reduce traumatic and ischemic damage in a number of experimental systems [12]. TLM has been shown to decrease the posttraumatic increase in blood-brain barrier permeability observed after head injury in rats [12, 13]. As shown by in vitro and in vivo studies, the efficacy depends on a combination of mechanisms: (1) The molecules of TLM insert within the lipid bilayer of the cell membrane, scavenging toxic lipid-peroxyl radicals and reducing the production of highly reactive hydroxyl radicals [12]; (2) the insertion of TLM into the cell membrane decreases the membrane phospholipid fluidity and thereby mitigates the progression of lipid peroxidation as it spreads across the membrane [12]; and (3) TLM increases the concentration of vitamin E, an important endogenous lipophilic antioxidant [14].

Administration of 21-aminosteroids in experimental endotoxemia, sepsis, and meningitis showed improvement of systemic and hemodynamic parameters, attenuation of inflammation, preservation of tissue oxygenation, and protection of endothelial cell integrity [5, 14]. In our model, animals become invariably bacteremic in the course of the disease and are thus prone to sepsis, which likely contributes to mortality [7]. TLM, in contrast to the other antioxidants examined, significantly attenuated the spontaneous mortality in this study, in keeping with the known benefits of the drug in sepsis models.

None of the 3 antioxidants studied here attenuated CSF inflammation, as measured by the concentration of $\text{TNF-}\alpha$ in CSF. Previous studies found that neurons in the dentate granule cells of the hippocampus undergo apoptosis during experimental bacterial meningitis, independent of ischemic damage. This process is mediated in part by $\text{TNF-}\alpha$ [15]. The lack of an effect of the antioxidant drugs on CSF concentrations of $\text{TNF-}\alpha$ is congruent with the lack of neuroprotection in the hippocampus.

In summary, using a model of bacterial meningitis caused by *S. pneumoniae*, we demonstrated a beneficial effect of treatment with the antioxidants NAC, DFO, and TLM on cortical injury, whereas the drugs were not efficacious in preventing hippocampal injury. Thus, while antioxidant strategies are clearly promising for the adjunctive therapy of bacterial meningitis, an

ideal drug should act at the level of both the cortex and the hippocampus.

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